Use of Sequential Staining to Characterize Post-translational Modifications

Ivan C. Gerling 1,Jian Wu 2,Michael J. Pabst 3and Nataliya I. Lenchik 2, (1)Deparment of Medicine, and The Center of Genomics and Bioinformatics, University of Tennessee Health Science Center, VAMC Research 151, 1030 Jefferson Avenue, Memphis, TN 38104, (2)Medicine, University of Tennessee Health Science Center, VAMC Research 151, 1030 Jefferson Avenue, Memphis, TN 38104, (3)Peridontology, University of Tennessee Health Science Center, VAMC Research 151, 1030 Jefferson Avenue, Memphis, TN 38104

One of the most useful aspects of two-dimensional (2D) gel electrophoresis for proteome characterization is its ability to resolve post-translationally modified protein entities into separate spots that can be individually measured. The only requirement is that the modification changes the relative mass or isoelectric point of the protein sufficiently to resolve it into a new spot on the 2D-gel system used to analyze the samples. Even on broad pI range gels we have often found sufficient resolution for phosphorylations and glycosylations to indeed observe separate spots. To take full advantage of this information from 2D-gels the individual spot most be characterized not just as to what protein(s) they contain but also whether or not they are indeed post-translationally modified. The mass spectrometry methods used to identify the protein(s) in a spot from a 2D-gel are excellent, but have some serious disadvantages when it comes to determining if a protein is or is not modified. The presence of, or neutral loss of, unique mass unit "signatures" from a peptide fragment can be used as positive confirmation of the presence of a specific modification and, indeed, mass spectrometry may be used to identify the exact amino acid in a protein that has been modified. Unfortunately it is almost impossible to determine with any degree of certainty that a protein in a spot is NOT modified. It is exceedingly rare that all peptide fragments of a peptide can be recovered and sometimes modification are lost to detection (from peptide fragments or full size proteins) due to a lack of stability under conditions and methods used for mass spectrometry.

An alternative approach for determining whether a protein spot on a 2D-gel is modified, is to stain only proteins altered by individual types of post-translational modification with specific dyes. Direct staining methods for detection of phosphoproteins have been developed. Unfortunately, the detection sensitivity and specificity of the older dyes is not very good and they have only gained limited use. A new phosphoprotein specific fluorescence-dye called "Pro-Q ™ Diamond" recently became available from Molecular Probes (Eugene, OR, USA). It was reported that this dye can be used with isoelectric focusing gels, 2-D gels or standard SDS-PAGE to detect phosphorylated tyrosine, serine, or threonine residues [1]. The same company also released two new fluorescence dyes for detecting glycoproteins, Pro-Q ™ Emerald 300 and Pro-Q ™ Emerald 488 glycoprotein gel stains. These dyes appeared to provide an attractive alternative to the labeling with radioactive sugars that had previously been used for specific glycoprotein detection. Our goal was

to use these new fluorescent dyes to develop a protocol for the sequential detection of phosphoproteins, glycoproteins, and all proteins in a single gel. This would allow us not only to visualize protein spots in 2D-gels with a sensitive protein stain, but also to make a preliminary assignment of specific post-translational modifications to each spot detected. Because phosphorylation and glycosylation play critical roles for protein function, such a simple and fast assignment of these modifications to specific protein spots could help focus 2D-gel proteome investigations to some of the most functionally important proteins.

Based on preliminary experiments to evaluate effectiveness of staining, destaining and flouresence signals at different laser excitation and bandpass emission filters for detection we designed a protocol for sequentially determining and capturing images of the phosphoproteome, glycoproteome and proteome from a single 2D-gel. First the gel was fixed in 250 mL fixation solution (50% methanol, 10% acetic acid) for at least 90 minutes and washed with distilled water twice. Then gels were incubated with 250 mL Pro-Q ™ Diamond phosphoprotein dye in the dark for 3-4 hours, and destained with four changes of destain solution (15% 1,2-propanediol, 50 mM sodium acetate, pH 4.0, 60 min per wash, last destaining step overnight). The phosphoproteome image was acquired on a laser scanner using a 532 nm laser excitation and a 555 nm bandpass emission filter. Following this image acquisition, the gel was stained for glycoproteins with Pro-Q ™ Emerald 488. First the gel was washed twice in 3% glacial acetic acid for 20 min and then incubated in oxidizing solution for 1 hour. After an additional washing of the gel 3 times with 3% glacial acetic acid, it was incubated in Pro-Q Emerald 488 dye solution for 2 hours or overnight in the dark. Then the gel was washed twice with 500 mL 3% glacial acetic acid before scanning. The glycoproteoms image was acquired using a 488 nm laser excitation and 530 nm bandpass emission filter. Next, the gel was stained with SYPRO Ruby a general protein stain (Molecular Probes). The gel was incubated with gentle agitation in SYPRO Ruby dye staining solution for 3 hours to overnight in the dark, then washed in 10% methanol and 7% acetic acid for 30 min. The total proteome image was acquired on a laser scanner using excitation at 488 nm and a 555 nm bandpass emission filter. Finally, the gel was also stained by a silver-staining method and the resulting gel images were captured at 300 dpi using a regular office scanner.

The potential interference of the dyes applied early in the process with the dyes applied late was evaluated by running in parallel identical samples either subjected to all staining steps, or only SYPRO Ruby stain, or only silver staining. The number of spots in the SYPRO Ruby images of a gel subjected only to SYPRO Ruby stain was only slightly higher than the number of spots in the SYPRO Ruby images of a gel subjected to the sequential staining procedure (456 vs. 397) suggesting only a minimal interference. Furthermore, 98% of the spots in the sequentially stained image were also present in the gel only stained with SYPRO Ruby. Suggesting that the preceding stains did not carry over to create new spots in the SYPRO Ruby image. With silver stain we detected 295 spots in the sequential staining image and 303 spots in the gel only stained with silver. The phosphoproteome stain also did not appear to interfere with the glycoproteome stain because

we found an almost identical number of spots in the glycoproteome image regardless of whether the sample had or had not first been stained with Pro-Q ™ Diamond. In the sequentially stained gel the total number of spots in each of the 4 images were: 361, 61, 397 and 356 for the phosphoprotein, glycoprotein, SYPRO Ruby and Silver stains, respectively. Only 15 spots in the silver stain images appeared not to have a matching spot in the SYPRO Ruby image, indicating that very little additional information had been gained by adding the silver staining step to the sequential staining.

Based on our comparison of autoradiography images of 32-P culture labeled (4h) samples with the Pro-Q ™ Diamond image of the same samples it is clear that the stain may produce a substantial number of false positive stained spots. The two images shared 138 spots, whereas 105 spots appeared exclusively in the autoradiography and 66 spots exclusively in the Pro-Q ™ Diamond stain image. These 66 spots are likely to represent a mix of false positive spots and phosphorylated proteins that did not incorporate the radioisotope well within the 4 h culture period.

A well-published concern regarding the identification of 2D-gel spots by mass spectrometry is the possibility that the protein identified may not represent the protein responsible for the variability in spot intensity observed across a set of gels (samples). This is particularly likely to happen when a more abundant protein that does not stain well with a dye is appearing at the same place on the gel as a less abundant protein that stains very well with the stain. In the images from sequential staining we have found that certain spots appearing in the phosphoproteome or glycoproteome images did not appear to have a counterpart in the SYPRO Ruby image suggesting a higher staining sensitivity for the modifications than for the protein in itself. This does raise the possibility that even in cases of matching spots the protein detected and identified based on the SYPRO Ruby image may be at the same place as a much less abundant and heavily modified protein, which is the one visualized in the image for the post-translational modification. Although all the proteins that we have identified that according to our stains were modified have had support in the literature for potentially being subject to that type of modification, we cannot rule out that this type of false assignment of modifications can occur. There are two approaches to reduce this problem. First if the assignment of a modification with high accuracy is essential for the data interpretation it should be confirmed by direct mass spectrometry evidence of the additional mass corresponding to the hypothesized modification. Second if the dataset is one of multiple samples with spot intensity differences between gels, then it is possible to analyze the SYPRO Ruby images and the Pro-Q ™ dye images separately and evaluate the changes in spot intensity across the sample set for each of the dyes. If there is significant correlation between the spot intensities produced by each of the dyes then it is highly likely that the spot in the SYPRO Ruby images represent the modified protein.

We have developed a protocol that allows us to conduct a quick and preliminary assignment of glycosylation or phosphorylation status to a protein spot on a 2D-gel. Although we would not recommend the use

of these dyes as a method to prove that a specific protein in a 2D-gel is modified, we believe it is an excellent tool for systems biology experiments.

References:

1. Martin K, Steinberg TH, Goodman T, Schulenberg B, Kilgore JA, Gee KR, Beechem JM, Patton WF. Strategies and solid-phase formats for the analysis of protein and peptide phosphorylation employing a novel fluorescent phosphorylation sensor dye. Comb Chem High Throughput Screen. 2003 Jun;6(4):331-9.

2. H art C, Schulenberg B, Steinberg TH, Leung WY, Patton WF. Detection of glycoproteins in polyacrylamide gels and on electroblots using Pro-Q Emerald 488 dye, a fluorescent periodate Schiff-base stain. Electrophoresis. 2003 Feb;24(4):588-98.

3. Wu J, Lenchik NJ, Pabst MJ, Solomon SS, Shull J, Gerling IC. Functional characterization of two-dimensional gel-separated proteins using sequential staining.